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PRECOCIOUS TERMINATION OF DIAPAUSE IN NECK- AND ABDOMEN-LIGATED PUPAL PREPARATIONS OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Abstract

When ligatures were placed between the head and thorax of freshly pupated hornworms, the resulting brainless preparations initiated adult development weeks earlier than intact diapausing controls and months earlier than similar preparations from which the brain had been surgically extirpated. This phenomenon could not be reproduced by removal of any single recognized cephalic neural or endocrine organ or any combination of organs. A similar accelerated development took place in many isolated pupal abdomens prepared by ligature or surgical section between the thorax and abdomen.

Extirpation of the prothoracic glands of either diapause or non-diapause pupae resulted in only a very slight delay in the onset of adult development. Nevertheless, this development remained highly dependent on the brain, since preparations lacking brains as well as prothoracic glands underwent a prolonged developmental arrest. Preparations lacking prothoracic glands demonstrated elevated levels of ecdysone at the outset of adult development, although these levels were slightly lower than those of intact individuals at a similar stage.

These findings suggest that sources of ecdysone outside the prothoracic glands can respond to a hormonally active brain and contribute significantly to the elevated titers of ecdysone that accompany much of normal adult development. In addition, the present results direct attention to the possible existence of a head-centered mechanism for maintenance of the low ecdysone titer necessary for the persistence of pupal diapause.

Introduction

Insect pupal diapause is classically conceived as a developmental hiatus attributable to the failure of the prothoracic glands (PG) to secrete ecdysone—a failure considered in turn to reflect the diapausing brain's inability to secrete the prothoracicotropic hormone (PTTH). But in the tobacco hornworm, *Manduca sexta,* the diapause-like arrest that ensues after surgical excision of the pupal brain is eventually terminated, albeit with substantial delay relative to intact pupae (Judy, 1972; Wilson and Larsen, 1974; Safranek and Williams, 1980). Possible mechanisms for this outcome include autonomous secretion of ecdysone by the PG or by additional tissues not subject to the classical type of regulation by the brain.

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Abbreviations: PG = prothoracic gland; PTTH = prothoracicotropic hormone; LD = long-day; SD = short-day; SEG = subesophageal ganglion; PTG = prothoracic ganglion; FG = frontal ganglion; MTG = mesothoracic ganglion; VNC = ventral nerve cord segment; CC-CA = corpora cardiaca-corpora allata complexes.

For forty years the PG have been considered the principal source of ecdysone. Nevertheless, ecdysteroid production in at least certain species has been shown to proceed outside the PG in sources that include the testes, ovaries, and oenocytes (for reviews, see Hoffman and Hetru, 1983; Rees, 1985). The physiological significance of these abdominal sources is supported by instances in which ecdysone-dependent development takes place in the absence of the PG–for example, after surgical extirpation of the PG or in isolated abdomens (Chadwick, 1955; Ichikawa, 1962; Hsaio *et al.*, 1975; Delbecque *et al.*, 1978; Delbecque and Sláma, 1980; Sláma, 1983). But most studies to date fail to clarify whether these sources contribute significantly to the rising levels of ecdysone that accompany molting in intact insects.

The brain is known to be an important and sometimes necessary organ for the generation of molt-inducing levels of ecdysone. But again, numerous instances of ecdysone-dependent development in the absence of the brain have been described (see Safranek and Williams, 1980, for review). The current study focuses on the pupal stage of the tobacco hornworm where we have encountered the paradoxical finding that decapitation of freshly pupated diapause-destined individuals actually accelerates the initiation of adult development. The present experiments document this finding and probe its endocrine basis, drawing attention to previously unsuspected aspects of the regulation of normal adult development.

Materials and Methods

Hornworms were reared as described previously (Safranek and Williams, 1980) at 25°C under either a short-day (SD, 12L:12D) or long-day (LD, 17L:7D) photoperiod. SD pupae were derived from larvae reared under SD conditions and normally underwent a pupal diapause. LD pupae were derived from larvae under LD conditions; these did not diapause but initiated development typically within five days after pupation. The day of pupation is termed "Day 1" of the pupal stage, and the first seven days of the pupal stage "Week 1." Operations and ligations were carried out as described previously (Safranek and Williams, 1980; Safranek *et al.*, 1980) and as described below. All operated preparations were maintained under SD conditions at 25°C, the initiation of development being recognized by detachment and retraction of the trachea from the overlying pupal cuticle of the forewings.

Removal of the subesophageal ganglion (SEG) or the prothoracic or mesothoracic ganglia (PTG, MTG) was accomplished through a small ventral midline incision beginning at the base of the pupal proboscis; in many instances the proboscis was still quite flexible and could be bent slightly from the midline to facilitate the surgical approach. Removal of the prothoracic glands (PG) was accomplished after removal of a rectangular section of the dorsal thoracic cuticle. The main body of each PG was identified nested in its characteristic position just medial to the large tracheal trunk adjoining the prothoracic spiracle. It was gently teased free of its fine connections to surrounding tissues and then withdrawn by the aid of two forceps used in a hand over hand fashion to draw the remainder of the gland slowly from more anterior regions. Abdomens were prepared by ligation within 6 h of larval-pupal ecdysis shortly after the pupal wings attained their final size and position. Older abdomens were surgically isolated using a razor blade to section the pupal cuticle and body wall behind the thorax. The gut was either ligated with a fine sterile thread or draped over the side of the exposed edge of the abdomen. A sterile glass cover slip was applied over the anterior end of the abdomen and sealed with melted wax (Tackiwax, CENCO). Radioimmunoassays of ecdysteroids were performed as described elsewhere (Carrow *et al.*, 1981).

Results

Effects of brain removal and head-ligation on the development of pupae

In the course of studies on the regulation of pupal diapause we noted that pupae head-ligated promptly after eclosion to the pupal stage initiated development much sooner than would have been expected of brainless pupae. To document this we ligated 100 SD pupae within 2–6 h after pupation. An additional 100 SD pupae were set aside as unoperated controls. All individuals were examined at weekly intervals for the onset of tracheal apolysis. As documented in Figure 1, the ligated preparations initiated development an average of one month prior to intact diapausing pupae and approximately four months earlier than expected for pupae whose brains had been surgically extirpated (Safranek and Williams, 1980).

We repeated the experiment on approximately 50 freshly ecdysed SD pupae and 50 similar LD pupae, with 50 intact SD pupae serving as controls. Here again over 50% of the neck-ligated preparations had initiated adult development by the fifth week, irrespective of LD or SD status; by the ninth week over 90% had done so. By contrast, 50% of the intact SD controls initiated development only after 11 weeks and 90% only after 13 weeks. The development of the LD preparations thus was delayed by more than a month relative to that of intact LD pupae, which, as previously mentioned, initiate development within a week of pupation. But both LD and SD preparations developed in this instance over a month before intact diapausing pupae and several months earlier than would have been expected of pupae whose brains had been excised rather than removed by neck-ligation.

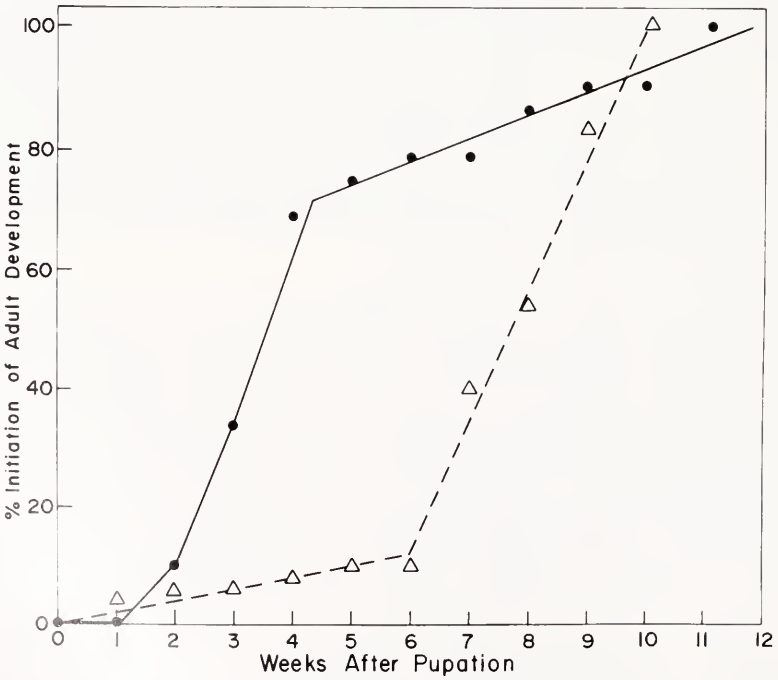

FIGURE 1. Acceleration of diapause termination by neck-ligation of diapause pupae. Diapause pupae were ligated 2–6 h after ecdysis. Initiation of development was recognized by apolysis of the wing epidermis. Ligated preparations are indicated by dark circles, intact controls by clear triangles.

Two explanations for these results seemed possible. Either a developmental inhibitor existed in the head which could be removed by ligation but not by brain excision or else the ligation itself provided an ecdysiotropic stimulus to the headless preparations. We examined these possibilities in the following experiments.

Effects of removal of the brain plus other cephalic organs

We attempted to identify a cephalic inhibitor by surgical extirpation of known cephalic neuroendocrine centers. Because head-ligated preparations lacked the brain as well as all other cephalic organs and because surgical extirpation of the brain alone produced a greatly prolonged diapause that could be readily distinguished from the abbreviated diapause of head-ligated preparations, most of our experiments involved simultaneous removal of the brain in addition to one or more of the other cephalic organs. Thus, the brain was removed in tandem with the subesophageal ganglion (SEG), the frontal ganglion (FG), the prothoracic ganglion (PTG), the mesothoracic ganglion (MTG), or the corpora cardiaca-corpora allata complexes (CC-CA). In addition, brain removal was performed in combination either with severance of all neural connections to the SEG, which was thereby left wholly free in the head, or with severance of the neural connection between the SEG and PTG only. Additional groups of brainless pupae with appropriate sham operations were established as controls. These experiments included operations on the PTG and MTG because of the proximity of these ganglia to a neck-ligature. All these experiments were performed on diapause-destined SD pupae 6 to 12 h after pupal ecdysis.

The results (Table I) demonstrated no significant differences between the various groups of pupae. Irrespective of the surgical procedures performed, all groups entered an extended diapause averaging over 30 weeks.

We considered in addition the possibility that the brain itself might be the source of an inhibitor. We were driven to entertain this possibility because for reasons of technical ease we routinely head-ligated pupae about 2 to 6 h after pupal ecdysis but normally surgically removed the brain 6 to 12 h after ecdysis. Thus, the brain could potentially have released an inhibitor during the initial 6 h after ecdysis. To investigate this possibility we removed the brains from a group of larvae 1–2 days prior to the wandering period; as controls we sham-operated additional larvae. Subsequently late on the day of pupal ecdysis we removed the brains of half of the previously sham-operated individuals. In addition, we sham-operated all the other individuals, both those that had previously lost their brains and also the other half of the previously sham-operated group. We subsequently monitored all the preparations for the initiation of development at weekly intervals. All of the 23 twice sham-operated preparations initiated adult development after 1–4 months. By contrast, brainless pupae entered an extended diapause regardless of whether the brain had been removed before ($n = 17$) or after pupation ($n = 23$). At 4 months less than 25% of either group had initiated development, and after 7 months more than 50% of each group still remained in diapause. Thus, evidence for a brain-derived inhibitor was entirely negative.

Effects of PG removal on adult development

All these results failed to define an inhibitory center in the head. This raised the possibility that head-ligation itself might produce an injury reaction which elicited development through a stimulatory effect on the PG. To examine this possibility we implanted into brainless, diapausing 1-day-old pupae the PG removed from 3-day-old SD pupal preparations that had previously been head-ligated on Day 1. As controls

we implanted the PG from intact Day 3 SD pupae into brainless 1-day-old pupal hosts and thereafter examined all the preparations for development at weekly intervals. During the subsequent 6 weeks, only 1 of the 15 pupae in each group initiated development. This contrasted greatly with the rapid development of a further control group of 18 head-ligated preparations in which half had initiated development by 6 weeks. These findings indicated that head-ligation did not generate within three days of ligation any irreversible, significant increase in PG activity.

In the preceding experiment, after removal of the PG from the head-ligated pupae, the thorax of each was resealed and the preparation observed at weekly intervals for the initiation of development. Much to our surprise, extirpation of the PG failed to block development of these decapitated preparations. At six weeks similar percentages of head-ligated pupae (9 of 18) and head-ligated pupae lacking PG (8 of 15) had terminated diapause, suggesting that development of head-ligated pupae did not require ecdysone secretion by the PG.

We initially considered that our surgical procedure must not have removed the entire PG. But repeated, detailed dissection of pupae after PG extirpation failed to reveal any retained portion of the PG. Comparison of the extirpated PG with whole PG carefully dissected from sacrificed pupae did not suggest that our routine operation left behind any portion of the PG.

These concerns were rendered moot by additional experiments. We prepared over 300 isolated pupal abdomens either by ligation within 4 h of the larval-pupal ecdysis or by surgical isolation of slightly older pupal abdomens as described under Materials and Methods. Although abdomens prepared by either approach had a very low mortality within the first week, preparations of both types experienced a sharply increasing mortality thereafter. Few preparations exhibited spontaneous motion beyond eight

TABLE I

Effects of surgical extirpation of cephalic and thoracic organs on diapause termination

Organs removed[1]	Number of preparations	% Developed at 50 weeks	Average time to development[2]
Brain plus sham[3]	181	67	35 ± 12
Brain plus SEG	41	85	34 ± 10
Brain plus PTG	27	81	31 ± 10
Brain plus FG	21	38	33 ± 12
Brain plus MTG	11	73	32 ± 11
Brain (SEG loose)	22	73	34 ± 12
Brain plus VNC[4]	17	65	39 ± 12
Brain plus CC-CA	16	78	36 ± 10

[1] Abbreviations: SEG: = subesophageal ganglion; PTG = prothoracic ganglion; FG = frontal ganglion; MTG = mesothoracic ganglion; VNC = ventral nerve cord; CC-CA = corpora cardiaca-corpora allata complexes.

[2] Averages are ± standard deviation and are calculated only for those preparations which had developed by 50 weeks. Thus the average time to development for the entire group of preparations would have been longer than that listed here.

[3] A group of sham-operated pupae was established for each experimental group of preparations listed below. No experimental group developed at a rate significantly different from its control group or from the pooled set of sham-operated preparations as listed on this line. Shams included brain removal plus a sham operation on the ventral thorax except in the control group for preparations without the brain and CC-CA complex, in which instance only the brain of the sham preparations was removed through a cephalic incision and no ventral incision was placed.

[4] The segment of the ventral nerve cord between the SEG and PTG was severed.

weeks. Nevertheless we repeatedly witnessed the spontaneous development of both male and female abdomens prepared by either technique: the earliest fully scaled and pigmented adult abdomen was obtained five weeks after pupation, others after as long as six months. Overall we have witnessed the adult development of 27 pupal abdomens, with approximately equal numbers of males and females and an average time from the isolation of the abdomens to the completion of adult development of 12 weeks. If survival could be enhanced, our experience suggests that many more, and perhaps all, of these preparations would be able to undergo adult development. The time course of development of these abdomens suggested that an abdominal source of a molting hormone could account for the spontaneous development of head-ligated pupal preparations: although we could not witness the initiation of adult development in isolated abdomens, the first individuals to complete adult development among either the head-ligated preparations or the isolated abdomens did so after about five weeks. Development took place in abdomens isolated from both LD and SD pupae; although the sample size was relatively small, no differences were noted in the times at which development was completed within the two groups.

What might this unusual source of molting hormone contribute to normal development? As we have seen here and elsewhere (Safranek and Williams, 1980), surgical extirpation of either the LD or SD pupal brain results in a developmental arrest of several months duration. By contrast, extirpation of the PG fails to block development of otherwise intact pupae. This was demonstrated in the case of LD pupae after removal of the PG from 12 Day 2 pupae: all of these pupae subsequently initiated development within 6 weeks of the operation, the earliest at 1 week, and 75% by 2.5 weeks. This represented a delay of only about 1 week relative to 12 control pupae whose PG had been extirpated and then replaced into the thoracic cavity, all of which initiated adult development within 6–11 days of the operation. PG removal also failed substantially to alter the duration of diapause in SD pupae. In this instance PG were removed from 18 Day 3 SD pupae; 15 similar pupae served as controls after receiving sham operations. Half of the 15 controls had initiated development at 9 weeks, as had half of the preparations lacking PG. The controls had an average diapause duration of 12 weeks, the experimentals, 13 weeks. Neither preparations subjected to PG extirpation nor sham-operated controls eclosed successfully; nevertheless, the course of adult development appeared grossly similar for both groups and essentially normal in its character and duration.

These experiments demonstrated the ability of an ecdysteroid source outside the PG to initiate and support adult development. In addition, the more rapid development of LD preparations relative to their SD counterparts suggested that these sources could respond to a hormonally active brain. Further to demonstrate this phenomenon we implanted brains from Day 1 LD pupae into Day 3 SD pupae from half of which both the brain-CC-CA complex and PG were removed, from the other half, the brain-CC-CA complex only. Both groups developed promptly. Of 10 preparations that had retained their PG, all commenced development within 6 weeks, the average being 2 weeks. Among the group without PG 3 of 14 developed only after several months whereas the remaining 11 initiated development within 5 weeks, the average of these being 3 weeks. Manifestly, the absence of the PG failed to block or even to delay substantially the rapid onset of development in response to a LD brain. In an additional control group of 15, the brain-CC-CA complex and the PG were removed but a LD brain was not implanted. All of these preparations underwent a prolonged diapause of at least three months and more than half remained in diapause after six months. As indicated in the previous experiments, these data again demonstrate that development can be stimulated by a hormonally active brain even in the absence of PG.

Is the development of preparations lacking PG accompanied by elevated ecdysteroid titers?

We inquired whether the onset of adult development in pupae lacking PG was accompanied by a rise in the ecdysteroid level. To this end we collected hemolymph samples from 3 groups of pupae on the day of tracheal apolysis—namely, intact LD pupae, LD pupae whose PG had been removed 24–48 h after pupation, and SD pupae whose PG had been removed at 2 weeks after pupation and which at that time had also received an implantation of a Day 1 LD pupal brain. We also measured the ecdysteroid levels of a group of 2–4 week old diapausing SD pupae. Samples were analyzed in a RIA for ecdysteroids. The results are summarized in Table II. Manifestly, both sets of preparations lacking PG had ecdysteroid levels markedly greater than those typically found in the course of diapause. Nevertheless, these titers were only about one-fourth those occurring in the intact LD pupae at a similar developmental stage.

Discussion

The present results describe a peculiar abbreviation of the hornworm pupal diapause when head-ligation rather than surgical excision was employed for removal of the brain promptly after pupal ecdysis. Adult development was advanced by months relative to surgically debrained preparations and by weeks relative to intact diapausing pupae. Yet we were unable to identify a cephalic source of a molting inhibitor among recognized neural or endocrine organs in the head.

We nevertheless continue to favor an inhibitor as an explanation of this phenomenon. Although we cannot rule out the involvement of a powerful stimulatory injury effect as can sometimes be seen after surgical manipulation of mature pupae (Wilson and Larsen, 1974; pers. obs.), several arguments oppose this explanation and favor an inhibitor. First, the considerable surgical injuries resulting from combined brain and SEG-PTG removal had no stimulatory effect on development, nor did we find evidence of any irreversible activation of the PG after ligation. Indeed, our experience suggests that injury shortly after pupation delays development: this is the case with young non-diapausing pupae where even a small cephalic cuticular wound will delay development by 1–2 days. Second, the developmental stimulation witnessed following neck-ligation of pupae does not occur promptly as one might expect from injury; rather the stimulation becomes apparent only after at least two weeks and often much longer. Third, the active involvement of an inhibitor during diapause would account for the ability of hornworm pupae to develop in the absence of the brain: the eventual disappearance of the hypothetical inhibitor would permit resumption of development even in the absence of a positive stimulus from the brain. These considerations lead

Table II

Effects of prothoracic gland extirpation on the ecdysteroid titer at the outset of adult development

Procedure	Stage	Ecdysteroid titer[1]
SD pupa-PG + LD brain	Tracheal apolysis	5.9 ± 1.9
LD pupa-PG	Tracheal apolysis	4.9 ± 1.8
LD pupa, intact	Tracheal apolysis	22±3
SD pupa, intact	Pupal diapause, 2 weeks	0.21 ± 0.03

[1] The ecdysteroid titer is expressed in μg/ml β-ecdysone equivalents.

us to suggest that in the normal course of the hornworm's pupal diapause an inhibitor is elaborated by the cephalic region that is responsible for the maintenance of the diapausing condition. Over time this inhibitor is eliminated by breakdown and/or by cessation of its production, thereby permitting the initiation of adult development. This model, consistent with the present findings, finds some support in the literature.

Results similar to those recorded here have been noted in *Antherea pernyi* (Waku, 1959) wherein pupae head-ligated promptly after pupation experienced an abbreviated diapause relative to both brainless preparations and intact unchilled diapause pupae. So also, in *Pieris brassicae* removal of the brain during diapause accelerated diapause termination; moreover, abdomens isolated from chilled diapausing pupae underwent spontaneous development in contrast to the persistent diapause of abdomens isolated from young, unchilled individuals (Kono, 1977). The acknowledged preeminent role of a molt-inhibiting hormone in the development of Crustacea illustrates the potential for inhibitory control of the ecdysteroid titer among arthropods (Highnam and Hill, 1977). These examples, as well as our own, do not preclude a contributing role for the brain in diapause termination. Indeed, we suggest that pupal-adult development may reflect an interplay of both stimulatory and inhibitory factors.

The final noteworthy finding of our study is the ability of isolated pupal abdomens to initiate adult development. This propensity is unique to this stage, since isolated larval abdomens, even when implanted with brains, fail to undergo a molt (Safranek and Williams, 1980). Development of isolated abdomens or of preparations lacking prothoracic glands has previously been noted in late larval and pupal stages of diverse orders of insects (Chadwick, 1955; Hsiao *et al.*, 1975; Delbecque *et al.*, 1978; Delbecque and Sláma, 1980) including the Lepidoptera (Ichikawa, 1962; Kono, 1977; Sláma, 1983). Production of ecdysteroids outside the PG has been even more widely documented and has been shown to derive from testes (Loeb *et al.*, 1982), ovaries (see Hoffman and Hetru, 1983, for review), or oenocytes (Romer *et al.*, 1974; Studinger and Willig, 1975). We do not presently know the source of molting hormone driving the development of isolated pupal hornworm abdomens, but since this development occurred in both male and female abdomens on a similar time scale, we favor an extra-gonadal source.

The contribution of these abdominal sources to normal adult development is uncertain. The present experiments make clear that sources of ecdysteroids outside the PG are more active in the presence of a non-diapausing brain. Moreover, in either diapause or non-diapause pupae these sources can support development in the absence of PG at a rate very nearly that of intact pupae. When development ensues in the absence of PG, ecdysteroid levels rise into a range near that of intact pupae. We note that development of preparations without PG does typically lag that of controls by several days and that the ecdysteroid titers at the outset of tracheal apolysis are uniformly slightly lower than those of controls at the same stage of development. Nevertheless the extra-PG sources clearly respond to an active brain and can generate high developmentally effective levels of ecdysteroids at least 25-fold greater than those found during the course of diapause. Thus, our observations indicate that at least one novel source of ecdysone plays a definite and possibly important role in the endocrine events normally associated with the onset of adult development.

Although the ecdysteroid source outside the PG responds to an active brain, it may differ from the PG in other aspects of its regulation. We note in particular that the time courses of development initiation in head-ligated preparations with or without PG as well as of isolated abdomens all appeared quite similar. Thus, the peculiar accelerated development of neck-ligated diapausing pupae could potentially reflect the activity largely of an abdominal ecdysteroid source. This source might be especially responsive to the disappearance of an inhibitory influence emanating from the head.

Manifestly the picture presented here differs substantially from that derived through classic studies of the Cecropia silkworm. Whether one or the other picture will prove paradigmatic we cannot yet say. But certainly the literature portrays a complex picture even among the silkworms. We have already noted the work of Waku in *Antherea pernyi*. In addition, Ichikawa (1962) described the development of isolated abdomens of *Samia cynthia* in the presence of an active brain. And the regulation of diapause termination in unchilled or inadequately chilled Cecropia or in Cecropia that fail to develop promptly after termination of chilling but which ultimately develop all remain to be investigated. Although the present experiments along with those of others make clear that the regulation of pupal diapause termination in the Lepidoptera will very likely not make for a simple yarn, even now we see evidence of a common thread.

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